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FACTORS AFFECTING THE SEPARATION OF MILK PROTEINS AND PARTIALLY HYDROLYZED PROTEINS BY CONTINUOUS FLOW PAPER ELECTROPHORESIS

The use of continuous paper electrophoresis in separating proteins has been described by GRASSMANN AND HANNIG[1], by DURRUM *et al.*[2,3], KARLER *et al.*[4], and by BRATTSTEN[5]. In these reports, instruments were described for continuous separation of proteins by paper electrophoresis. Several such instruments of this type are now commercially available. Most of the work on separations thus far reported has been on serum proteins and been concerned with the separation into fractions rather than in the production of pure fractions.

It has been reported by ZWEIG AND BLOCK[6] and MACRAE AND BAKER[7] that casein could be separated into its three components by zone paper electrophoresis. The latter authors determined the relative amounts of the three proteins by determining the amounts of bound dye or nitrogen analysis for each separate band.

The advantage of the continuous method over the zone method is that larger amounts can be separated and further tests of homogeneity can be made on the separated fractions.

BRATTSTEN[5] found that the resolving power of the cell he constructed was about one mobility unit. If this degree of separation of proteins is attainable, then it should be possible to separate α-, β-, and γ-casein by this method, which have mobilities of -6.8, -3.0, -2.0 units respectively at pH 8.4 and 0.1 ionic strength. The present paper describes the separation of these three caseins and the essential conditions of purifying proteins by this method.

MATERIALS AND METHODS

The continuous flow paper electrophoresis cell used was the Model CP manufactured by Beckman Instruments, Inc., Belmont, Calif.**.

Analysis of fractions from the curtain were made by measurements of biuret color, absorption at 278 mμ, or use of FOLIN-LOWRY reagent[8]. Biuret determinations

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* Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

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on fractions were made using the reagent of BEISENHERZ[9]. 6 ml of biuret reagent was mixed with 1 ml of sample and after 30 min, the absorbance was read at 574 mμ against a reagent blank.

Absorbance at 278 mμ was measured in Beckman Model DU spectrophotometer using 1 cm quartz cells. Since 0.025 ionic strength veronal has considerable absorbance near 278 mμ, a reagent blank was used.

Free electrophoresis was conducted in the 2 ml cells of the Spinco Model H Electrophoresis-Diffusion apparatus. The buffer used was pH 8.4, 0.1 ionic strength, 0.05 in veronal and 0.05 sodium chloride as previously used for the casein fractions in this laboratory[10].

β-Casein was prepared following the urea method of HIPP, GROVES AND MCMEEKIN[10]. The trypsin hydrolysis was conducted as described by PETERSON, NAUMAN AND MCMEEKIN[11].

Casein for the fractionation experiments was prepared by an acid precipitation at pH 4.6, followed by an extraction at pH 4.0 with acetic acid as described in a recent publication by MCMEEKIN *et al.*[12]. By this method, a number of materials which amount to only 1 % of the total are removed.

Bovine plasma albumin, lot R370295A, was manufactured by Armour and Company.

EXPERIMENTAL

The Spinco Continuous Electrophoresis instrument is enclosed in a large plexiglass case to prevent rapid evaporation from the paper curtain. It was operated in a cold room at 5°, and chilled buffer from an 8 l vessel was circulated through a cooling plate ¼ inch behind the curtain before it was pumped into a reservoir at the top of the apparatus. An overflow diverted the largest part of this flow back into the reservoir. The height of the buffer in this reservoir governs the rate of feed by the upper siphon curtain which supplied buffer to the lower curtain where the electrophoretic separation is effected. The electrophoretic separation is made on a large sheet of heavy filter paper whose lower edge is divided into 32 drip points for the 32 collection tubes. At a point near the upper edge, the sample is applied, and the flow of buffer down the sheet carries the fraction into the collection tubes. The anode and cathode are two large packs of filter paper on the right and left edges of the lower sheet. In order to carry away the electrolysis products, two siphons carry buffer from the upper reservoir onto the top of these electrodes.The buffer flows down these electrodes and is recirculated.

As a preliminary to these experiments, a sheet of the filter paper supplied with the instrument was hung and wet with pH 8.6, 0.025 μ veronal buffer. At 1 inch intervals marked with pencil dots, a grid was constructed. After closing the face of the apparatus, equilibrium was established for 3 h, then with the buffer still flowing, a speck of solid dye was applied at each spot. At a downward flow rate of 8.0 ml/h (divided into 32 fractions), the side siphons were then adjusted for the most even collection in the sample tubes. The dye markers could not be used to follow buffer flow after voltage was applied to the sheet.

An estimate of spreading of a pure protein on the sheet was then obtained with a 2 % bovine plasma albumin (BPA) solution in 0.025 μ pH 8.6 veronal buffer. With no voltage applied to the sheet, a thin trace of protein ran down from the point of application to the drip points and all the protein was contained in one of the 32 sample collection tubes. To make the trace visible for photography, the curtain was removed from the apparatus and quickly dried at 110° in a forced draft hot air oven. Then it was stained by the usual paper electrophoresis procedures using bromophenol blue[13].

A major variable is the rate of addition of the sample to be separated. The same degree of separation can be achieved by using repeated runs at a high sample addition rate, then taking a narrow fraction and rerunning the material. In this work we tried to determine the maximum separation obtainable by one passage down the curtain.

Bovine plasma albumin was used as the test substance to determine the effect of rate of feed on zone width. Using 0.01 μ pH 1.8 buffer, 6 % acetic acid, 1 % formic acid, a 2 % solution of bovine plasma albumin, which had been previously dialyzed against the buffer, was put on the curtain. Fig. 1 shows the results. At a downward flow rate of 15.4 ml/h, 410 V at 30 mA produced a migration of about 18 cm (20 drip points). If the feed rate was 2 mg (0.1 ml) per h, the width of the trace expanded to 6 tubes; 6 mg (0.3 ml) per h widened the zone to 10 tubes. This shows clearly that for single pass separation, the feed rate must be kept low.

To find the relation between evaporation of buffer on the sheet and current flow, a low initial flow rate was used. With no voltage applied, 7.8 ml of buffer came down the curtain per hour. As the voltage and current were increased stepwise, the heat dissipated in the curtain finally rose to 65 W. At this point (84 mA and 780 V), the total flow had decreased to 6.4 ml/h, but the flow in the tubes was uneven, and the middle tube was dry.

Since the veronal in the buffer is non-volatile, 25 ml portions of the buffer and the samples were evaporated at 110°. The surprising results are shown in Table I.

TABLE I

Current through curtain mA	*Buffer flow ml/h*	*Av. conc. of buffer μ*
14	8.9	0.025
56	7.8	0.052
84	6.4	0.095

Evidently the wicks on the side of the curtain are acting as a source of buffer to replace the water loss from the heated curtain. Approximately four times as much buffer volume is entering the sheet as is dripping from the lower points. The use of this much current will greatly distort the separations since the lateral flow of buffer into the sheet will compress the pattern and oppose the migration due to the mobility of the components.

The choice of working conditions may then be summarized. For the best single passage separation, flow of buffer down the curtain is set at the lowest value which

does not distort the pattern of separation. Voltage (and wattage) are kept low, below 20 watts. Feed rate is held at about 2–4 mg of protein per hour.

The separation of a 50–50 mixture of α-lactalbumin and β-lactoglobulin on the acidic or basic side of the isoelectric point is a good test of the resolving power of the

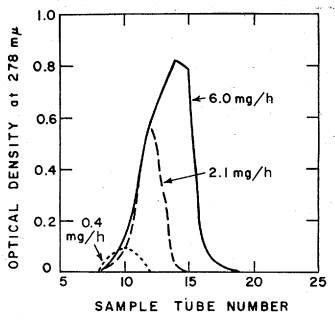

Fig. 1. Effect of sample feed rate on spread of bovine plasma albumin in collection tubes.

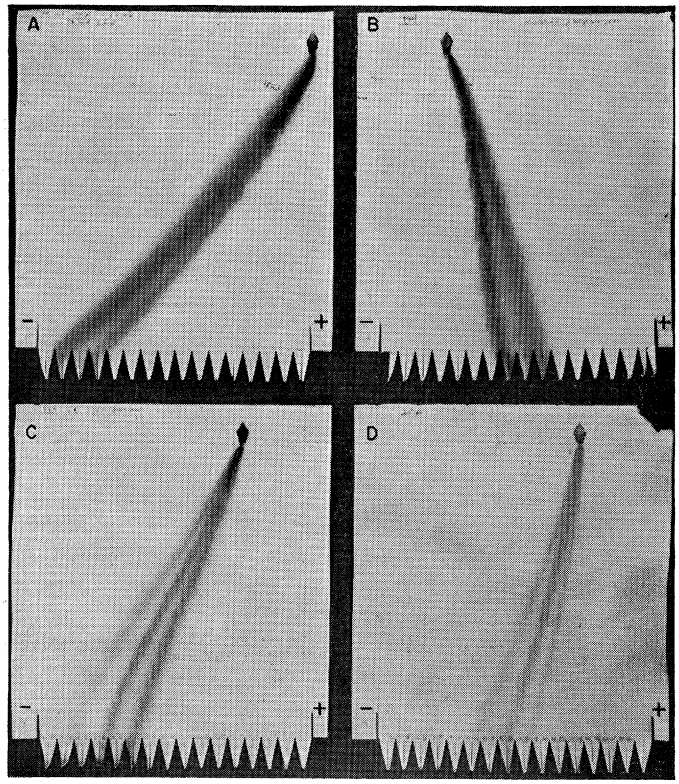

Fig. 2. (A) Separation of α-lactalbumin from β-lactoglobulin at pH 1.8, 0.01 μ. (B) Separation of α-lactalbumin from β-lactoglobulin at pH 9.0, 0.015 μ. (C) Separation of β-casein trypsin hydrolysate at pH 1.8, 0.01 μ. (D) Separation of β-casein trypsin hydrolysate in 40 % urea pH 2.0, 0.01 μ.

apparatus. At pH 1.8, in 0.01 μ acetic acid–formic acid buffer, the two proteins have to flow nearly across the sheet before any separation is visible. On the alkaline side, a lesser flow is sufficient. The tabulated mobilities for free electrophoresis are as follows:

TABLE II

MOBILITIES OF β-LACTOGLOBULIN AND α-LACTALBUMIN IN ACID AND BASIC BUFFERS AT 0.1 μ

	pH 2.0	*pH 8.5*
β-Lactoglobulin	+ 10	— 5.0
α-Lactalbumin	+ 7.8	— 4.2

These indicate that the separation should be easier at pH 2.0 than 8.5. However, the reverse is true, as shown in Fig. 2A and B. The quality of the separation at pH 9.0 in 0.015 ionic strength triethylamine carbonate buffer is shown in Fig. 3 where the ultraviolet absorption of the sample collection tubes is plotted. Of 100 mg of the mixture, 36 and 35 mg were collected in tubes 15–18 and 20–24. Paper strip electrophoresis of the lyophilized products showed that the separation was clean.

Although the values were obtained for 0.1 ionic strength solutions, the same ratio of mobilities exist in the 0.01 ionic strength pH 2 buffer used on the curtain. The

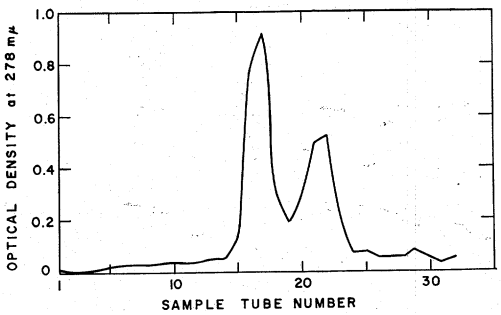

Fig. 3. Separation of α-lactalbumin and β-lactoglobulin in pH 9.0, 0.015 μ triethylamine carbonate buffer.

mobility of β-lactoglobulin in this buffer was measured at $14.7 \cdot 10^{-5}$ cm/sec/V, that of α-lactalbumin at $11.5 \cdot 10^{-5}$ cm/sec/V. Neither of the proteins showed any abnormalities in shape of the migrating peaks.

The behavior of the same protein mixture at too high a feed rate is shown in Fig. 4A. At 1 ml/h feed, 20 mg of protein mixture was put on the curtain per hour. This large amount of α-lactalbumin and β-lactoglobulin showed no separation in the sample tubes and the curtain showed only a large blur after drying and staining. This agreed with our spreading tests with a single protein.

The separation of α-, β-, and γ-casein was next attempted. At 3 mg/h feed rate, 0.025 ionic strength veronal buffer at pH 8.6, and a downward flow rate of 13 ml/h, α-casein migrated 15 tubes from the starting point, and β-casein 7 tubes from the starting point. This is with a potential of 300 V at 17 mA (Fig. 4B).

This could be improved further, as shown in Fig. 4C. Here the counterosmotic flow was shown by adding 1 % lactose to the 2 % casein feed solution. On drying, the lactose reacted with the buffer to produce a tan color which was outlined in pencil to show in the photographs. Here the feed rate was reduced to 2 mg/h and the cleanness of the separation was improved.

Finally, as shown in Fig. 4D, the downward flow of buffer was decreased to 10 ml/h with other conditions the same. Here owing to the counterosmotic flow, the paths

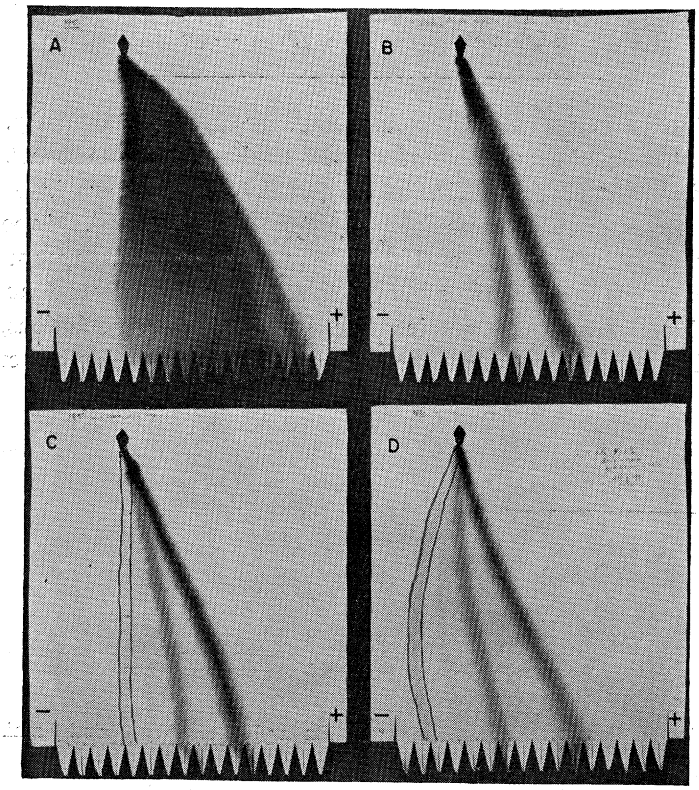

Fig. 4. (A) Flooding of curtain at 1 ml/h feed rate. (B) Separation of whole casein into α-, β-, and γ-fractions, feed 3 mg/h. (C) Same separation, feed 2 mg/h. (D) Same separation, buffer flow lowered to 10 ml/h.

of the migrating proteins were distorted from straight lines. Further decrease in buffer flow would result in the proteins being swept into the lefthand side-electrode.

When whole casein is analyzed by zone electrophoresis at pH 8.6 in 0.05 ionic strength veronal buffer, the apparent mobility depends on the paper wetness. In a thin paper such as Whatman No. 1, the mobility is about 54 % and in the thicker Whatman No. 3 paper, the mobility is about 71 % of the value in free solution. The curtain is thicker than Whatman No. 3 and is wetter, so the mobility may be expected to be higher.

The mobility on the curtain can be calculated if we know the field strength in

volts/cm and the length of time the protein is in the electrical field on the curtain[14]. From the rate of buffer flow into the sample tubes and the average buffer content of the curtain (determined by weighing), it appears that the protein is in the field for 6 h. This agrees with the time for the first appearance of protein in the sample tubes after the protein is started on the curtain. This 6 h holds, of course, only at the buffer flow rate of 10.6 ml (in 32 tubes) and for the applied voltage of 300 V at 17 mA. With the front of the cabinet removed after the curtain had been running for some time, a vacuum tube voltmeter was used to measure the actual voltage distribution on the curtain. When the voltmeter on the power supply indicated 300 V, the actual voltage difference between the sides of the curtain at the top was 170 V, while at the bottom it was 233 V. This was distributed linearly across the 30 cm width of the curtain between the filter paper electrodes. The mobility calculations are summarized in Table III.

TABLE III

COMPARISON OF FREE ELECTROPHORESIS MOBILITIES AND MOBILITY ON THE CURTAIN AT pH 8.6, 0.025 IONIC STRENGTH

	α-Casein	*β-Casein*
Curtain	$9.0 \cdot 10^{-5}$	$4.8 \cdot 10^{-5}$
Free, descending	10.05	5.01
Free, ascending*	16.7	11.1

* The ascending boundary mobilities are obviously abnormal since the δ-boundary is much larger.

The use of the volatile buffer, triethylamine carbonate[15], at pH 9.0 enabled a comparison between the apparent composition by analysis of areas in free electrophoresis and the actual weight recoveries. The casein used had been given an extraction with 1 % acetic acid which removes less than 1 % by weight of the casein, containing a number of interfering materials[12]. Its electrophoresis diagram by fringe analysis showed 72 % α-casein, 26.3 % β-casein, and 1.7 % γ-casein. WARNER[16] first demonstrated the complexing of β-casein in the α-casein peak area.

When fed onto a curtain at a high feed rate, 4 mg/h and a downward buffer flow of 15 ml/h, 360 V sufficed to spread the protein out nearly across the sheet. The buffer was 0.015 ionic strength and pH 9.0 triethylamine carbonate. The current was only 18 mA. As the buffer did not absorb UV light at 278 mμ, a comparison could be made of weight recovery and UV absorption using the extinction coefficients supplied by HIPP[17] of 1.024, 0.497, and 0.500 for α-, β- and γ-casein respectively.

The weight recoveries after the flow paths had been established for three days period are given in Table IV.

MACRAE AND BAKER[7] have shown that whole casein, analyzed by paper electrophoresis after correcting for the different dye binding capacities, consisted of 60 % α-casein, 32 % β-casein and 9 % γ-casein. MCMEEKIN, HIPP AND GROVES[12] have shown similar weight recovery of α-casein in a quantitative separation.

	% of total		
	α-casein	β-casein	γ-casein
Weight recovery*	58.6	35.4	6.0
UV absorption	57.8	37.5	5.8
Area analysis of starting material	72.0	26.3	1.7

* After adjusting to pH 4.6 with dilute acetic acid, the fractions were lyophilized in weighed flasks, then dried 16 h *in vacuo* at 40°.

It must be noted that these results were obtained after the curtain was saturated with the casein fractions. Earlier runs were lower in α-casein. Fig. 5 represents the UV absorption of the material in the collection tubes. The purity of the lyophilized α- and β-casein fractions are shown in Fig. 6 as the electrophoresis diagrams in pH 8.6, 0.1 ionic strength buffer.

In this laboratory we have made a preliminary chemical fractionation of the peptides formed from β-casein by the action of trypsin[11]. After centrifuging off the insoluble material formed at pH 7.0 after 20 min of trypsin action, the solution is

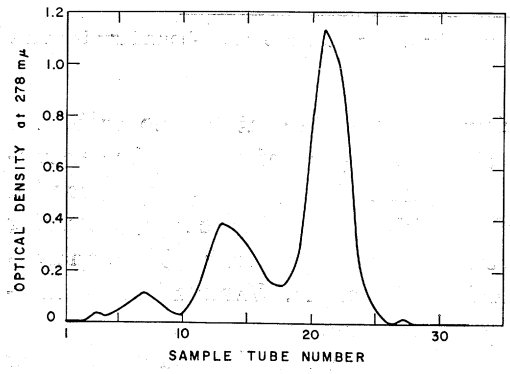

Fig. 5. Separation of α-, β-, and γ-casein using pH 9.0, 0.015 μ triethylamine carbonate buffer.

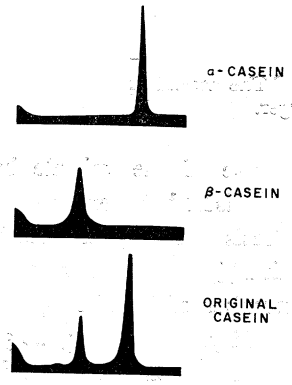

Fig. 6. Free electrophoresis of α- and β-casein fractions separated by curtain electrophoresis.

acidified to pH 4.7. The precipitate at this point amounts to about 20 % of the starting material and contains 5 major component peptides with mobilities — 1.8, — 2.4, — 3.0, — 5.1, and — 9.4 at pH 9.0, 0.1 ionic strength in the Tiselius apparatus.

If this material is separated on the curtain using a 1 % formic acid–6 % acetic acid buffer, at a downward flow of 7 ml/h (total in 32 tubes) 240 V at 17.5 mA gives a visible separation of four components on the dyed sheet (Fig. 2C). The phosphopeptone, — 9.4 mobility, dissolves off the sheet in the bromophenol blue dyebath. However, on subjecting the lyophilized fractions to analytical paper electrophoresis, it is seen

that only two components, mobility — 1.8 and — 9.4, have been obtained pure. This is despite the apparent cleanness of the separation as shown by analysis of the protein content of the tubes with biuret reagent (Fig. 7). GRANZER[18] has shown that the products of chymotryptic digestion of insulin also are complex although they migrate as single lines. By using 40 % urea or 4 % phenol in the acid buffer, he was

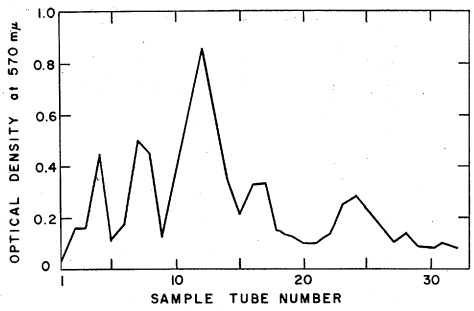

Fig. 7. Separation of peptides produced from β-casein by trypsin action, pH 1.8, 0.01 μ.

able to separate the mixture of peptides with one operation. Use of the urea as a solvent in the curtain apparatus reduces the number of tubes over which the fractions were spread, so the first fractionations were not successful. However, by still further reducing the downward flow of buffer, peptides with mobilities of — 2.4 and — 3.0 were obtained in a pure state.

It is also possible to purify the other peptides by repeated passage down the curtain. For instance, fraction — 2.4 was obtained in electrophoretically pure form after three passages down the curtain at pH 8.6, 0.025 ionic strength.

SUMMARY

The conditions for the separation of proteins by curtain electrophoresis have been determined. At low rates of sample application, proteins with a difference of one unit of mobility can be separated unless a high degree of interaction exists.

This method is shown to be particularly effective in separating α-, β-, and γ-casein, a mixture of α-lactalbumin and β-lactoglobulin, as well as peptides produced by the action of trypsin on β-casein.

The amounts of material which can be purified by curtain electrophoresis are small, however, they are adequate to produce enough material for characterization of proteins and peptides by modern analytical methods.

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